

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/66, 15/13, 15/70, 15/73</b>		A1	(11) International Publication Number: <b>WO 97/09436</b> (43) International Publication Date: 13 March 1997 (13.03.97)
(21) International Application Number: PCT/AU96/00555		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 5 September 1996 (05.09.96)			
(30) Priority Data: PN 5239 5 September 1995 (05.09.95) AU			
(71) Applicant (for all designated States except US): CRC FOR BIOPHARMACEUTICAL RESEARCH PTY. LTD. [AU/AU]; 384 Victoria Street, Darlinghurst, NSW 2010 (AU).			
(72) Inventors; and		Published	
(75) Inventors/Applicants (for US only): VANCOV, Tony [AU/AU]; 2A Mulla Road, Yagoona, NSW 2199 (AU). ZAHRA, David [AU/AU]; 6 Yew Place, Quakers Hill, NSW 2763 (AU). HAWKINS, Nicholas, John [AU/AU]; 20 Moncur Street, Woollahra, NSW 2025 (AU). WARD, Robyn, Lynne [AU/AU]; 20 Moncur Street, Woollahra, NSW 2025 (AU).		With international search report.	
(74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).			

## (54) Title: METHOD FOR PRODUCING PHAGE DISPLAY VECTORS

## (57) Abstract

The present invention consists in a method for producing a phage display vector which includes causing or allowing recombination between (i) a first vector which includes a sequence encoding a first polypeptide chain of a specific binding pair member; and (ii) a second phage vector which includes a sequence encoding cre-recombinase operatively linked to a control sequence which allows expression of the Cre recombinase and a sequence encoding a second polypeptide chain of a specific binding pair member; wherein the recombination event gives rise to a recombinant phage vector in which expression of the Cre recombinase is substantially inhibited. The present invention also consists in a phage vector which includes a nucleic acid sequence encoding a promoter sequence, a first recombination site downstream and adjacent to the promoter sequence and an open reading frame for cre-recombinase positioned downstream and adjacent to the recombination site such that the promoter drives expression of the Cre recombinase.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## **METHOD FOR PRODUCING PHAGE DISPLAY VECTORS**

The present invention relates to phage display vectors and to methods for producing recombinant phage display vectors.

Functional antigen-binding domains of antibodies (heavy and light chain variable domains) can be displayed on the surface of filamentous bacteriophage. International patent application WO 93/19172 describes methods, recombinant host cells and kits for production of antibodies displayed on the surface of phage. To produce a library of great diversity, recombination occurs between two vectors comprising nucleic acid encoding immunoglobulin light and heavy chains respectively producing a recombinant vector encoding the two polypeptide chains. Antibodies displaying the desired antigen binding specificity can be selected from the large number of clones produced by a process called panning.

The size of antibody libraries generated using most phage display systems is limited by the low transformation efficiency of *Escherichia coli*. As the size of the mammalian antibody repertoire is estimated to be in the order of  $10^6$  to  $10^8$ , methods for the generation of larger libraries are required.

A number of groups have investigated the possibility of increasing the size of antibody libraries by combinatorial infection. In principle, heavy and light chains within the initial library or from the original single chain libraries have been systematically shuffled to obtain libraries of exceptionally larger numbers. Two different mechanisms of site-specific recombination have been used to achieve the association of the two libraries:

1. The *lox-Cre* system of bacteriophage *P1* (Waterhouse *et al.*, 1993; Griffiths *et al.*, 1994; WO 93/19172)

In essence, *E.coli* is transformed with a repertoire of heavy chain antibody genes (encoded on a phagemid). When Cre recombinase is provided *in vivo* by infecting the *E.coli* with *P1*, the heavy chains residing on the vector and phagemid are exchanged via the *lox-P* sites. Chain exchange is, however, reversible. Other disadvantages of using this system include a dependency on infection with a Cre-encoding phage and lack of selection for recombination.

2. The att recombination system of bacteriophage lambda (Geoffroy *et al.*, 1994)

This process makes use of lambda phage att recombination sites and the Int recombinase to irreversibly create a chimera between plasmid and phagemid vectors carrying respectively, variable light and variable heavy sequences. *E.coli* is transformed with a repertoire of light chain antibody sequences (encoded on a phagemid). Selection of the recombinant phagemid is possible by the assembly of a chloramphenicol resistance marker upon the correct recombinational event. These features represent certain advantages over the lox-Cre system, however, contrary to the authors' findings, vectors possessing two functional *E.coli* origins of replication are inherently unstable. The fact the att recombination system results in the creation of a potentially highly unstable recombinant, detracts from using it to generate large antibody libraries.

A major problem of the methods used to date is that the recombination process is reversible, therefore stable recombinants are not produced. Furthermore, the methods are dependent on infection with a Cre-expressing bacteriophage and the recombinant phagemid only contains one *E.coli* replicative origin. Furthermore, the methods used to date do not allow the easy selection of recombinants.

Accordingly, in a first aspect the present invention consists in a method for producing a phage display vector, which method includes causing or allowing recombination between

- (i) a first vector which includes a sequence encoding a first polypeptide chain of a specific binding pair member; and
- (ii) a second phage vector which includes a sequence encoding Cre recombinase operatively linked to a control sequence which allows expression of the cre recombinase and a sequence encoding a second polypeptide chain of a specific binding pair member;

wherein either the first or second polypeptide chain is fused to a component of a replicable genetic display package which thereby displays the fused polypeptide at the surface of replicable genetic display packages; and

wherein the recombination event gives rise to a recombinant phage vector which includes sequences encoding both the first and second polypeptide chains and in which expression of the Cre recombinase is substantially inhibited.

By "operatively linked" we mean that a nucleic acid sequence is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operatively linked to a coding sequence if it affects the transcription of the sequence.

5 In a preferred embodiment of the first aspect of the invention, the second phage vector includes a nucleic acid sequence encoding a promoter sequence, a first recombination site downstream and adjacent to the promoter sequence and an open reading frame for cre-recombinase positioned downstream and adjacent to the recombination site, such that the promoter allows expression of the Cre recombinase sequence; and wherein 10 recombination takes place at the first recombination site such that the open reading frame for Cre recombinase is separated from the promoter sequence.

In a further preferred embodiment the promoter sequence is derived 15 from a promoter for a gene encoding a selectable marker. Preferably, the selectable marker is a gene encoding resistance to an antimicrobial agent. Preferably, the antimicrobial agent is chloramphenicol. In a further embodiment, the promoter is an inducible promoter. The inducible promoter may provide increased control over initiation of the recombination event.

20 In a further preferred embodiment the second phage vector also includes a terminator sequence positioned downstream of the Cre recombinase open reading frame in order to prevent undesired transcription of genes downstream of the Cre recombinase gene. The terminator may be the rrnbT1T2 terminator sequence.

25 In a further preferred embodiment of the invention, the first vector includes a first recombination site upstream and adjacent to a sequence encoding an open reading frame of a selectable marker, wherein recombination results in the positioning of the sequence encoding the open reading frame of a selectable marker on the recombinant phage vector adjacent to and downstream of the promoter sequence such that the promoter 30 sequence allows expression of the selectable marker. The selectable marker may, for example, be a gene encoding resistance to an antimicrobial gene, or a gene that reacts with a chromogenic compound such as the lacZ gene or the X-gal gene. In a preferred embodiment the selectable marker is a gene encoding resistance to an antimicrobial agent. Preferably, the antimicrobial 35 agent is chloramphenicol.

In a further preferred embodiment the first and second vectors each include a first recombination site and a second recombination site different from the first, site-specific recombination taking place between first recombination sites on the first and second vectors and between second recombination sites on the first and second vectors, but not between the first recombination site and the second recombination site on the same vector.

Preferably, the first recombination site is the *loxP* sequence. The second recombination site is preferably a mutant *loxP* sequence, more preferably the *loxP511* sequence.

10 In a preferred form of the invention the first vector is a plasmid and recombination takes place in a bacterial host.

The recombination event may give rise to a second recombinant vector which contains a sequence comprising the Cre recombinase open reading frame. In order to prevent expression of the Cre recombinase from this vector, a terminator sequence may be included upstream of the recombination site of the first vector used in the method of the present invention. This terminator sequence should prevent any undesirable expression of the Cre recombinase following the recombination event. This terminator sequence is preferably different to the terminator sequence, if present, on the original second phage vector. The terminator sequence may, for example, be the λ terminator.

20 Alternatively, in order to avoid exposure of the recombinant phage vector to Cre recombinase which may be expressed from this second recombinant vector, the recombinant phage particles may be isolated from the host organism.

25 In a further preferred embodiment of the invention, either the first or second vector further includes

- (a) a sequence encoding a detectable label;
- (b) an inducible stop codon; and
- (c) a sequence encoding an enzymatic cleavage site

30 such that the sequences described in (a) to (c) are included in the recombinant phage vector. Preferably, the detectable label is the c-myc peptide label, the inducible stop codon is the amber codon and the enzymatic cleavage site is the subtilisin cleavage site.

35 It will be appreciated that the detectable label allows immunological detection of expressed antibodies without the need to use antibodies against

the specific binding pair member. The inducible stop codon allows expression of the polypeptide chains as either a displayed fragment or as a soluble product by using either suppressor or nonsuppressor strains of *E.coli* for expression. The enzymatic cleavage site facilitates release of the phage from the specific binding pair member.

5 In a further preferred embodiment, the first vector is pUX-1 and the second phage vector is pMOX-1.

In a second aspect the present invention consists in a phage vector which includes a nucleic acid sequence encoding a promoter sequence, a 10 first recombination site downstream and adjacent to the promoter sequence and an open reading frame for Cre recombinase positioned downstream and adjacent to the recombination site such that the promoter drives expression of the Cre recombinase.

15 In a preferred embodiment of the second aspect of the present invention, the promoter sequence is derived from a promoter for a gene encoding a selectable marker. Preferably, the selectable marker is a gene encoding resistance to an antimicrobial agent. More preferably, the antimicrobial agent is chloramphenicol.

20 In a further preferred embodiment of the second aspect of the present invention the recombination site is the *loxP* sequence. Preferably, the vector further includes a second recombination site which is different to the first recombination site. The second recombination site is preferably a mutant of the *loxP* sequence, more preferably the *loxP511* sequence.

25 It will be understood that the method of the present invention may be used to produce phage display vectors which are suitable for preparing combinatorial libraries of antibodies. The specific binding pair member may therefore be an antibody or an antibody fragment. The first polypeptide chain may, for example, be an immunoglobulin heavy chain and the second polypeptide chain may be an immunoglobulin light chain, or vice versa.

30 The phage vector used in the method of the present invention is designed to transiently express the Cre recombinase until exchange between the two vectors occurs. In particular, the promoter is used to drive Cre recombinase gene expression until recombination occurs. The recombination event substantially silences expression of the Cre recombinase and therefore gives rise to a stable recombinant phage vector. In a preferred 35 form of the invention the recombination event results in an exchange of

genetic material between the first and second vectors such that the Cre recombinase open reading frame is transferred from the second phage vector to the first vector.

In a preferred form of the invention, the recombinant phage vector  
5 not only includes sequences encoding two separate polypeptide chains of a specific binding pair member, but also includes a functional selectable marker. This provides a means for selecting microorganisms containing recombinant phage vectors.

In order that the present invention may be more clearly understood, a  
10 preferred form thereof will be described with reference to the following examples and figures in which:

**Figure 1.** Diagnostic RE analysis of MCO5. Lanes 1 to 4 are undigested MCO5 clones 1 and 2, MCO1 and MCO3 respectively. Lanes 5 to 8 are SacI  
15 digested MCO5 clones 1 and 2, MCO1 and MCO3 respectively and indicates that MCO5 remains undigested whereas MCO1 and MCO3 are linearised. Lanes 10 to 13 are XbaI digested MCO5 clones 1 and 2, MCO1 and MCO3 respectively and indicates that MCO5 remains undigested whereas MCO1 and MCO3 are linearised. Lanes 14 to 17 are EcoRI digested MCO5 clones 1  
20 and 2, MCO1 and MCO3 respectively and indicates that all plasmids are linearised with this RE. Lane 9 is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

**Figure 2.** PCR amplification of the LacVH cassette from MCO5. Lanes 2 to 5 are the PCR products of MCO5 (lanes 2 and 3) and MCO3 (lane 4) and the no template control (lane 5) using MC19 and MC28 as primers. MCO5 produces a product of approximately 950bp and MCO3 produces a product of 2.5kb as expected. Lane 1 is lambda DNA digested with HindIII and EcoRI as  
25 molecular weight markers.

**Figure 3.** Diagnostic RE analysis of pBIISK-Pcat. Lanes 1 to 9 are mini prep clones 3, 4, 6 to 12 of pBIISK-Pcat digested with BglII and SalI. C is pBIISK(BglII) digested with BglII and SalI. The banding pattern indicates that all clones except clone 11 has the 250bp Pcat fragment cloned into pBIISK(BglII). M is lambda DNA digested with HindIII and EcoRI as  
30

molecular weight markers. Ms is pBluescript plasmid DNA digested with HaeIII as molecular weight markers.

5           **Figure 4.** PCR amplification of the Cre recombinase gene. Lanes 1 and 2 is the PCR products of p35Scre using primers MC25 and MC26. p35Scre produces a PCR product of ~1kb as expected. M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

10           **Figure 5.** Diagnostic RE analysis of clones of pBIISKcre. C is the control DNA [pBIISK(BglII)] digested with BssHII and XbaI. Lanes 3, 4, 5, and 7 are the respective clones of pBIISKcre digested with BssHII and XbaI as indicated. An XbaI digest was expected to linearise the control and clones. The expected fragment sizes for the pBssHII RE digest were 2.8kb, 861bp and 352bp for the clones and 2.9kb and 51bp for the control DNA. The banding pattern indicates that the Cre PCR product has been cloned into pBIISK(BglII). M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

15           **Figure 6.** Diagnostic RE analysis of clones of pBIISKterm. The reaction products of clones 1 to 5 are shown on the left panel and clones 6 to 10 in the right panel. Three diagnostic reactions were performed for each clone: a) digested with SalI and XbaI (left lane); b) PstI (middle lane); and c) undigested (right lane). The XbaI and SalI double digest releases the 380bp Term fragment as indicated. M is lambda DNA digested with HindIII and EcoRI as molecular weight markers. (Note that undigest DNA for clones 5 and 10 are not shown).

20           **Figure 7.** Diagnostic RE analysis of clones of pBIISK-TLVH. The control DNA [pBIISK(BglII) - lanes 1 and 2] and each clone (1, 3, 6, and 9 - lanes 3 to 8) were digested with SacI (left) and PstI, SalI plus BglIII (right lane). As expected the SacI digest linearises the plasmid and the triple digest releases the 950bp LacVH fragment and the 380bp Term fragment as indicated - these fragments are not present in the control DNA. M is lambda DNA digested with HindIII and EcoRI as molecular weight markers. Ms is pBluescript plasmid DNA digested with HaeIII as molecular weight markers.

**Figure 8.** Diagnostic RE analysis of pMOX. Clones 9 and 11 of pMOX were digested with XbaI and HindIII (lanes 1 and 2 respectively) which releases the 250bp Pcat fragment that is the same size as an XbaI and HindIII digest of pBIISKPcat-Cre (lane 3). Similarly, clones 9 and 11 of pMOX were digested with SalI and PstI (lanes 4 and 5 respectively) which releases the 380bp Term fragment that is the same size as a SalI and PstI digest of pBIISK-TLVH (lane 6). M is lambda DNA digested with HindIII and EcoRI as molecular weight markers. Ms is pBluescript plasmid DNA digested with HaeIII as molecular weight markers.

5

**Figure 9.** Diagnostic screening for pUX. Twelve clones were digested with PstI (left lane) and undigested (right lane) to screen for the inclusion of the LacVH fragment cloned into pUTcat. Lane C is similarly digested vector pUTcat. Clone 11 clearly has the lacVH fragment cloned into the PstI site since it is releasing a fragment of ~950bp that is a similar size to the LacVH PCR product (lane L) and which the control DNA does not have (left lane C). It is also clearly evident that the undigested supercoiled DNA is larger than the control DNA (right lane C). M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

10

**Figure 10.** Analysis of the orientation of the LacVH insert of pUX clone 11. pUX clones 10, 11 and 12 were digested with BalI (lanes 1, 2 and 3 respectively). A 700bp fragment is released for the correct orientation and 1120bp for the incorrect orientation. Lane 2 clearly indicates that the LacVH fragment in clone 11 is in the correct orientation. pUX clones 10, 11 and 12 were also digested with BglII (lanes 5, 6 and 7 respectively) and indicates that pUX is linearised as expected. M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

15

**Figure 11.** Diagnostic RE analysis of pUX-TT. The isolate clone of pUX-TT was digested with XhoI (lane 1), SpeI (lane 2), XhoI and SpeI (lane 3) and undigested (lane 5). The double digest releases the 600bp TT heavy chain DNA sequence. Lane 4 is XhoI and SpeI digested pUX. M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

20

25

30

35

**Figure 12.** Diagnostic RE analysis of pMOX-TT. Twelve clones (1 to 12) were digested with SacI and XbaI (left lane) and undigested (right lane) to screen for the inclusion of the TT light chain DNA fragment cloned into pMOX. Lane C is similarly digested vector pMOX. All clones clearly have the TT fragment cloned into the SacI and XbaI sites since a fragment of ~600bp is released which the control DNA does not have (left lane C). It is also clearly evident that the undigested supercoiled DNA for each clone is larger than the control DNA (right lane C). M is lambda DNA digested with HindIII and EcoRI as molecular weight markers.

10

**Figure 13.** Diagrammatic representations of pMOX (a) and pUX (b).

**Figure 14.** Diagrammatic representation of the proposed mechanism of *in vivo* recombination between the pMOX and pUX vectors.

15

**Figure 15.** Diagrammatic representation of the recombinant phage vector pMUX.

## EXAMPLES

### Construction of the Lac VH cassette

The LacVH cassette was based on the antibody expression vector MCO1 (Ward et al., 1996), which contains cloning sites for both the heavy and light chain DNA sequences. The first part of the construction of the cassette was to remove the XbaI restriction endonuclease (RE) site (3' cloning

site of the light chain) from MCO1. Following digestion with XbaI, MCO1 was treated with klenow fragment of DNA polymerase I (blunted) and subsequently religated and transformed into *E. coli* strain XL1-blue. Twenty transformants were selected and subjected to RE and gel electrophoretic analysis. Fifteen of these clones were found to be devoid of the XbaI site.

One construct, designated MCO4, was retained for further work. The second part of the construction was to remove the light chain leader region from EcoRI to SacI sites. MCO4 was digested with SacI, gel purified and redigested with EcoRI. The double cut MCO4 DNA was then subjected to klenow treatment to 'fill-in' the cohesive ends, ligated and transformed into XL1-blue. This construction procedure destroyed the SacI site but retains the integrity of the EcoRI site. Twenty transformants were randomly selected

20

25

30

35

and subjected to RE analysis with EcoRI, SacI and XbaI. At least 10 clones were isolated that displayed the expected profile. This construct is referred to as MCO5.

In order to positively determine whether these recombinants were devoid of the 86bp light chain leader sequence, the nucleotide sequence of this region was determined for four clones using MCO3.for (MC28) PCR primer. All four clones were found to be devoid of the said sequence and one clone was retained. Figure 1 indicates that MCO5 but not MCO1 and MCO3 (which has stuffer fragments cloned into the light and heavy chain cloning sites) are linearized by SacI and XbaI, while all three MCO vectors are linearized with EcoRI. As expected, it is also evident that MCO5 is slightly smaller than MCO1.

A 956bp fragment corresponding to position 90 and 987 of MCO5 was then amplified by the polymerase chain reaction (PCR) using primers MC19 and MC28. The resultant PCR product, designated as the LacVH cassette, contained starting from the 5' end, a unique PstI site, the lacZ promoter and operator sequence (lacZ P/O), the pel B leader sequence, XhoI and SpeI unique RE sites, a myc tag, an amber point mutation, a subtilisin open reading frame (ORF), the gene III ORF, a translational stop codon, a mutated loxP site (designated loxP511) and a unique BglII site [Figure 2].

The loxP511 site was introduced into the cassette with the reverse primer MC19, which incorporated a loxP511 DNA sequence. Intramolecular excision events have been noted where recombination occurs between two loxP sites that are in the same orientation of the DNA substrate (Abremski et al., 1986). In order to avoid this problem in the final construct (pMOX), and in the recombinatorial vector, the loxP511 sequence was positioned in the opposite orientation with respect to the wild type loxP site.

The amplified LacVH cassette was subsequently used in the construction of both the acceptor phage vector and the donor plasmid vector.

#### Construction of pMOX

This vector was based on MCO1 and was designed to transiently express 'cre' recombinase until an exchange between vectors has occurred. The chloramphenicol acetyltransferase (CAT) gene promoter (Pcat) is used to drive the cre-recombinase gene expression. Between the Pcat promoter and

the Cre gene is the loxP sequence which is one of two designated recombination sites.

Construction of pMOX involved the PCR amplification of four discrete fragments that were cloned into MCO1. These fragments are referred to as: Pcat, which contains the chloramphenicol promoter and the loxP site 3' of the promoter; Cre, which contains the sequences necessary to express the creatin recombinase protein; Term, which contains ribonuclease terminator sequences; and LacVH, which contains the cloning site for the heavy chain of an antibody and the loxP511 site 3' of the gene III stop codon.

10

#### Construction of the Pcat-Cre cassette

The Pcat element was successfully amplified from the CAT gene residing on the plasmid pACYC184 using the primers MC27 and MC17. To determine the efficacy of the amplified Pcat element and whether the loxP site interfered with transcription, the PCR product was cloned into the promoter-probed vector pkk232-8. Following blunting with Klenow to remove the terminal A's, the Pcat PCR product was digested with HindIII and cloned into the HindIII/SmaI site of pkk232-8. The ligation mix was used to transform XL1-blue cells and promoter efficiency determined by spreading the cells on LB agar plates containing varying (10-50mg) concentrations of chloramphenicol. Five Pcat clones were isolated and were subjected to PCR and gel electrophoretic analysis. All five were found to harbour the Pcat element (including the loxP site) indicating that the Pcat promoter was not compromised because of the presence of the loxP site.

Attempts to clone the Pcat element into the vector pBltSK(BglII) [kindly provided by Dr. Guy Lyons, Kanamatsu Laboratory, University of Sydney] using the unique XbaI and HindIII RE sites were unsuccessful. To facilitate cloning of the Pcat element, two further primers were designed (MC50 and MC51) that amplified the Pcat PCR product and also contained additional HindIII RE sites at both ends of the PCR product. The Pcat PCR fragment and pBltSK(BglII) were digested with HindIII RE, purified in an agarose gel, ligated, and used to transform XL1-blue cells. Twelve transformants were selected and subjected to RE and gel electrophoretic analysis. Eight clones were found to contain the Pcat PCR fragment and one clone was selected for further work and named pBltSK-Pcat. Figure 3

20

25

30

35

indicates that the Pcat fragment is released by HindIII RE digestion of this plasmid.

The cre recombinase ORF (including the ribosome binding site RBS) was amplified from pBS157 [kindly provided by Dr Peter Waterhouse, CSIRO Division of Plant Industry, Canberra, Australia] with primers that were designed using the E. coli P1 nucleotide sequence derived by Sternberg et al. (1986). These two primers (MC25 and MC26) incorporated unique HindIII and SalI RE sites. After successful amplification of the Cre fragment (Figure 4) the product was digested with HindIII and SalI, and ligated into HindIII and SalI digested pBltSK(BglII). The ligation mix was used to transform XL1-blue cells and eleven of 12 single colonies screened by RE analysis were shown to contain the Cre gene. Four clones further characterised by RE analysis showed the expected banding patterns (Figure 5). Clone 3 was designated pBltSK-Cre and used for further manipulations.

The Pcat-Cre cassette was constructed by cloning the Pcat fragment (from pBltSK-Pcat) into pBltSK-Cre. This was performed by digesting both pBltSK-Pcat and pBltSK-Cre with XbaI and HindIII. The 4kb fragment from pBltSK-Cre and the 250bp fragment from pBltSK-Pcat were agarose gel purified and ligated. The ligation products were used to transform XL1-blue cells and 12 single colonies were isolated. Initial screening indicated that 10 of the 12 clones had the Pcat fragment cloned into the pBltSK-Cre vector. Further diagnostic digests were performed on the clones and one was designated pBltSK-PcatCre and used in subsequent manipulation.

**25 Construction of Term-LacVH cassette**

In order to prevent read through and interference from Pcat, the E. Coli. 5s ribosomal transcription terminator (rrnbT1T2) [Brosius et al., 1981] was positioned adjacent to the 3' end of the Cre ORF. The rrnbT1T2 terminator was successfully amplified from pkk232-8 using the primers MC24 and MC23, generating a product of 340bp. The transcriptional terminator product was restricted with SalI and PstI and ligated into the cloning vector pSP72. The fragment was subsequently extracted from this vector by digestion with SalI and PstI, and ligated into PstI and SalI digested pBltSK(BglII). The ligation mix was used to transform XL1-blue cells and 12 single colonies were isolated and screened for the inclusion of the Term fragment. All 12 colonies contained the Term fragment (figure 6) and one

was selected, further characterized and designated pBltSK-Term. This plasmid was used for subsequent manipulations.

The next stage in acceptor phage vector construction was to clone the LacVH gene into pBltSK-Term, to produce the Term-LacVH cassette. As described above a 956bp fragment had been PCR amplified from MCO5. New primers (MC48 and MC49) were designed to extend the ends of that fragment, adding HindIII cloning sites to both ends. A fragment of appropriate size was amplifiable using these two primers and the original PCR product as template. The PCR product and pBltSK-Term were then digested with PstI and BglII, gel purified and ligated. The ligation reaction was used to transform XL1-blue cells and 12 single colonies were isolated and screened for the inclusion of the LacVH fragment. Eleven of the 12 colonies contained the LacVH gene. Further RE diagnostic digests were performed (Figure 7) and one clone was selected and designated pBltSK-TLVH. This clone was used for subsequent DNA manipulations.

#### Construction of pMOX

The final step in the construction of pMOX involved digesting: a) MCO1 with XbaI and BglII; b) pBltSK-TLVH with BglII and SalI; and c) pBltSK-PcatCre with SalI and XbaI. The MCO1 vector, TLVH and PcatCre cassettes were purified in an agarose gel, mixed and ligated. The ligation mix was used to transform XL1-Blue cells and 12 colonies were isolated and screened for the correct construction. Figure 8 indicates expected restriction digest patterns of the resultant pMOX vector.

#### Construction of pUX

Construction of pUX was based on pUC19 (Yanisch et al., 1985). Since both the donor and acceptor vectors possessed the same antibiotic resistance (ampicillin - ApR) determinant, the ApR gene of pUC19 was replaced with a PCR amplified Tetracyclin resistance (TcR). The entire TcR gene was amplified from pACYC184, using primers that included Pvul RE sites at both ends of the PCR product (MC21 and MC22). The TcR gene was restricted with Pvul, and ligated with the 1790bp Pvul restricted fragment of pUC19. This fragment was partially devoid of the ApR coding region (thereby making it sensitive to Ap), as well as the coding region for lacZ. The ligation mixture was then transformed into the Tc E. coli sensitive strain

NM522, and recombinants were selected for resistance to Tc. Following PvuI RE analysis one clone (designated pUT-1, 3209bp) was retained.

The remainder of the vector was constructed by cloning two PCR generated fragments into the MCS of pUC19. These fragments are referred to as: CAT (which contains the sequences that induce resistance to chloramphenicol); and LacVH (which contains the cloning site for the heavy chain of an antibody).

The chloramphenicol resistance gene (the ORF excluding its promoter region) was PCR amplified using primers MC18 and MC20. The cat gene primers were based on the nucleotide sequence of the cat gene of the plasmid Tn9 (Alton and Vapnek, 1979). After amplification the loxP-cat gene was restricted with SacI/PstI and ligated into SacI/PstI restricted pUT-1 vector. The ligation mixture was transformed into NM522 and mini-plasmid lysate preps of eighteen randomly chosen isolates were analysed by RE and gel electrophoresis. Additional characterization of four clones with PCR revealed that all four clones (designated pUTcat) contained, the loxP-cat gene.

The next step in the construction of pUX was to clone the lacVH gene into the PstI site. This was accomplished by amplifying the LacVH PCR fragment described earlier with primers (MC48 and MC58) that added a PstI site to both ends of the LacVH gene. The PCR fragment and pUTcat was restricted with PstI, the DNA was purified in an agarose gel and the DNA ligated. The ligation mixture was transformed into NM522 cells and mini-plasmid lysate preps of eighteen randomly isolated clones were analysed by RE and gel electrophoresis. One clone contained the DNA insert (Figure 9) which, with additional RE analysis, was shown to be in the correct orientation (Figure 10). This clone was designated pUX.

#### In vivo recombination of pUX and pMOX

To test for 'in vivo' recombination between pUX and pMOX, the heavy chain of a tetanus toxoid (TT) Fab was cloned into pUX, while the light chain was cloned into pMOX. Phage was then prepared from pMOX-TT, and was used to infect log phase HB2151 cells previously transfected with pUX-TT.

#### Construction of pUX-TT and pMOX-TT

The tetanus toxoid heavy chain was RE digested with XhoI/SpeI and purified in an agarose gel from MCO1-TT. This fragment was ligated into XhoI/SpeI RE digested pUX. The ligation mixture was used to transform HB2151 cells and a single colony was isolated. This colony was screened using RE digests and gel electrophoresis for the inclusion of the TT heavy chain DNA. Figure 11 indicates that this clone contained the TT heavy chain DNA sequences. The tetanus toxoid light chain was RE digested with SacI/XbaI and purified in an agarose gel from MCO1-TT. This fragment was ligated into SacI/XbaI RE digested pMOX. The ligation mixture was used to transform XL1-blue cells and 12 colonies were isolated and screened using RE digests and gel electrophoresis for the inclusion of the TT light chain. Figure 12 indicates that all 12 colonies contained the TT light chain DNA sequences. These two plasmids were referred to as pUX-TT and pMOX-TT.

15 Preparation of pMOX-TT phage.

pMOX-TT was transformed into XL1-blue and 250 $\mu$ l of an overnight culture was used to inoculate 2YT containing 2% glucose, carbenicillin (carb - 50 $\mu$ g/ml) tetracycline (tet - 10 $\mu$ g/ml) and then rescue with helper phage (VCS-M13). The culture was incubated for 2 hours at 37°C with shaking and then centrifuged at room temperature at 4500rpm for 15 minutes. The bacterial pellet was resuspended in 50ml of 2YT containing carb 50 $\mu$ g/ml, tet 10 $\mu$ g/ml and kanamycin 70 $\mu$ g/ml and incubated overnight at 37°C with shaking. The bacteria were pelleted by centrifugation at 8000rpm at room temperature. The phage were precipitated from the supernatant by adding 1/5vol 20% PEG6000/2.5M NaCl, mixing and leaving on ice with occasional shaking for 1 hour. The phage were collected by centrifugation at 8000rpm for 20 minutes at 4°C. The pellet was resuspended in 500 $\mu$ l of 1% BSA, and bacterial debris was removed by a high speed centrifugation in a microfuge tube and stored at 4°C.

30

In vivo recombination between pUX-TT and pMOX-TT

A 1ml culture of pUX transformed HB2151 cells in media containing tetracycline (OD600 = 0.8) were infected with 1x10<sup>9</sup> pMOX-TT phage and allowed to stand at 37°C for 30 minutes. Carb (25 $\mu$ g/ml) was added and the culture incubated for 30 minutes with shaking. Further carb (25 $\mu$ g/ml) was added and incubated with shaking for a further 3 hours. An aliquot of cells

35

added and incubated with shaking for a further 3 hours. An aliquot of cells (250 $\mu$ l) was obtained 1 and 4 hours after infection and plated onto: a) Carb; b) Tet; c) Chlor10; d) Chlor50; e) Carb/Chlor10; and f) Carb/Chlor50.

Since pMOX is tet sensitive and pUX is carb sensitive, growth of cells in culture indicated that the cells contain both pMOX and pUX.

5 Chloramphenicol further selects for cells that have recombinants containing the pMUX vector since chloramphenicol resistance is acquired only after recombination. A lawn of colonies were observed on both chlor and carb/chlor plates (at both concentrations of chlor) following infection of 10 pMOX-TT into the culture containing HB2151/pUX-TT indicating that recombination had occurred.

15 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

#### List of Primers

20 MCO3.for (MC28) 5'..ACC TGC AGA ACA GTG AGC GCA ACG CAA T..3'  
*PstI* site for cloning

MCO3.rev (MC19) 5'..GAA GAT CTT CAT AAC TTC GTA TAT ACA TAT  
GTA TAC GAA GTT AT G CGG CCG CTT AAA TTA ATT AGC..3'  
25 *BglIII* site for cloning and [*loxP*511] recombination site

Pcat.for (MC50) 5'CCC AAG CTT GGG CTC TAG AGC CGA CGC ACT TTG  
CGC CGA AT..3' Forward primer for Pcat PCR fragment; *XbaI* and  
30 *HindIII*

PcatPCR.rev (MC51) 5'..CCC AAG CTT CCC AAG CTT GGG ATA ACT TCG  
TAT AGC ATA CAT TAT..3' Reverse primer for Pcat PCR fragment  
35 with *LoxP* site; *HindIII*.

Pcat.for (MC27) 5'..GCT CTA GAG CCG ACG CAG TTT GCG CCG ATT..3'  
Reverse primer for the amplification of LacVH of pMOX.

Pcat.rev (MC17) 5'..GGG AAG CTT CCC ATA ACT TCG TAT AGC ATA  
CAT TAT ACG AAG TTA T GAT AAC TCA AAA AAT ACG  
40 CC..3' Reverse PCR primer for amplification of the CAT gene  
promoter fro Tn9. *HindIII* for cloning and [*loxP*]

Cre.rev (MC25) 5'..ACG CGT CGA CCG CGT TAA TGG CTA ATC GC..3'  
Reverse primer for the amplification of cre recombinase of pMOX.

Cre.for (MC26) 5'..CCC AAG CTT CTG AGT GTT AAA TGT CCA ATT  
TAC..3' Forward primer for the amplification of cre recombinase of  
45 pMOX.

Term.for (MC24) 5'..ACG CGT CGA CCA GAA GTG AAA CGC CGT A..5'

Forward primer for the amplification of terminator for pMOX.

Term.rev (MC23) 5'..TTC TGC AGT TCC TGA TGA TGC AAA AAC GAG

GC..3' Reverse primer for the amplification of terminator of pMOX.

5 MCO5.for (MC48) 5'..CCC AAG CTT GGG ACC TGC AGA ACA GTG AGC  
GCA ACG CAA T..3' Forward primer for PCR of LacVH of pMOX;  
*PstI, HindIII*

MCO5.rev (MC49) 5'..CCC AAG CTT GGG AAG ATC TTC ATA ACT TCG

TAT ATA CAT A..3' Reverse primer for PCR of LacVH with loxP  
site; *BglII, HindIII*.

tet.rev (MC21) 5'..TAC GAT CGT ATT CAC AGT TCT CCG CAA GA..3'

Reverse primer for the amplification of tet<sup>R</sup> for pUX.

tet.for (MC22) 5'..ATC GAT CGA TCA AAT GTA GCA CCT GAA GTC AG..3'  
Forward primer for the amplification of tet<sup>R</sup> for pUX.

15 cat.for (MC18) 5'..CGA GCT CGA TAA CTT CGT ATA ATG TAT GCT ATA  
CGA AGT TAT] GAT TTT CCA GGA GCT AAG GAA..3' Forward  
PCR primer for the amplification of the CAT resistance gene from  
Tn9; *SacI, [loxP]*

20 cat.rev (MC20) 5'..TTT GCA GAT CGT CAA TTA CCT CCA CG..3' Reverse  
primer for amplification of Cat<sup>R</sup> gene for pUX.

MCO.rev PPCR (MC58) 5'..AAA ACT GCA GCC AAG ATC TTC ATA ACT  
TCG TAT ATA CAT A..3' Reverse PCR primer for LacVH MCO PCR  
product; *PstI, BglII*

**BIBLIOGRAPHY**

Abremski, K., Frommer, B. and Hoess, R. H. (1986) Linking-number changes in the DNA substrate during Cre-mediated loxP site-specific recombination. Journal of Molecular Biology 192(1):17-26.

Alton, N. K. and Vapnek, D. (1979) Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9. Nature 282(5741):864-9.

Brosius, J., Dull, T. J., Sleeter, D. D. and Noller, H. F. (1981) Gene organization and primary structure of a ribosomal RNA operon from Escherichia coli. Journal of Molecular Biology 148(2):107-27.

Sternberg, N., Sauer, B., Hoess, R. and Abremski, K. (1986) Bacteriophage P1 cre gene and its regulatory region. Evidence for multiple promoters and for regulation by DNA methylation. Journal of Molecular Biology 187(2):197-212.

Ward, R. L., Clark, M. A., Lees, J., Hawkins, N. J. (1996) Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. J. Immunol. Methods. 189: 73-82.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33(1):103-19.

**CLAIMS:**

1. A method for producing a phage display vector, which method includes causing or allowing recombination between
  - 5 (i) a first vector which includes a sequence encoding a first polypeptide chain of a specific binding pair member; and
  - (ii) a second phage vector which includes a sequence encoding Cre recombinase operatively linked to a control sequence which allows expression of the cre recombinase and a sequence encoding a second polypeptide chain of a specific binding pair member;

10 wherein either the first or second polypeptide chain is fused to a component of a replicable genetic display package which thereby displays the fused polypeptide at the surface of replicable genetic display packages; and

15 wherein the recombination event gives rise to a recombinant phage vector which includes sequences encoding both the first and second polypeptide chains and in which expression of the Cre recombinase is substantially inhibited.
- 20 2. A method according to claim 1 wherein the second phage vector includes a nucleic acid sequence encoding a promoter sequence, a first recombination site downstream and adjacent to the promoter sequence and an open reading frame for cre-recombinase positioned downstream and adjacent to the recombination site, such that the promoter allows expression of the Cre recombinase sequence; and wherein recombination takes place at the first recombination site such that the open reading frame for Cre recombinase is separated from the promoter sequence.
- 25 3. A method according to claim 2 wherein the promoter sequence is derived from a promoter for a gene encoding a selectable marker.
- 30 4. A method according to claim 3 wherein the selectable marker is a gene encoding resistance to an antimicrobial agent.
- 35 5. A method according to claim 4 wherein the antimicrobial agent is chloramphenicol.

6. A method according to any one of claims 1 to 5 wherein the first vector includes a first recombination site upstream and adjacent to a sequence encoding an open reading frame of a selectable marker, wherein recombination results in the positioning of the sequence encoding an open reading frame of a selectable marker adjacent to and downstream of the promoter sequence such that the promoter sequence allows expression of the selectable marker.

5

10 7. A method according to claim 6 wherein the first vector includes a terminator sequence upstream and adjacent to the first recombination site.

8. A method according to any one of claims 1 to 7 wherein the first and second vectors each include a first recombination site and a second recombination site different from the first, site-specific recombination taking place between first recombination sites on the first and second vectors and between second recombination sites on the first and second vectors, but not between the first recombination site and the second recombination site on the same vector.

15

20 9. A method according to any one of claims 2 to 8 wherein the first recombination site is the *loxP* sequence.

10. A method according to claim 8 or claim 9 wherein the second recombination site is a mutant *loxP* sequence.

25

11. A method according to claim 10 wherein the second recombination site is the *loxP511* sequence.

30

12. A method according to any one of claims 1 to 11 wherein the first vector is a plasmid.

35

13. A method according to any one of claims 1 to 12 wherein recombination takes place in a bacterial host and the recombinant phage particles are isolated from the bacterial host.

14. A method according to any one of claims 1 to 13 wherein the specific binding pair member is an antibody or an antibody fragment.

15. A method according to any one of claims 1 to 14 wherein either the  
5 first or second vector further includes

- (a) a sequence encoding a detectable label;
- (b) an inducible stop codon; and
- (c) a sequence encoding an enzymatic cleavage site

such that the sequences described in (a) to (c) are included in the

10 recombinant phage vector.

16. A phage vector which includes a nucleic acid sequence encoding a promoter sequence, a first recombination site downstream and adjacent to the promoter sequence and an open reading frame for Cre recombinase positioned downstream and adjacent to the recombination site such that the  
15 promoter drives expression of the cre-recombinase.

17. A phage vector according to claim 16 which further includes at least  
one nucleic acid sequence encoding a polypeptide chain of a specific binding  
20 pair member.

18. A phage vector according to claim 17 wherein the specific binding pair member is an antibody or an antibody fragment.

25 19. A phage vector according to any one of claims 16 to 18 wherein the promoter sequence is derived from a promoter for a gene encoding a selectable marker.

20. A phage vector according to claim 19 wherein the selectable marker  
is a gene encoding resistance to an antimicrobial agent.  
30

21. A phage vector according to claim 20 wherein the antimicrobial agent is chloramphenicol.

35 22. A phage vector according to any one of claims 16 to 21 wherein the recombination site is the *loxP* sequence.

23. A phage vector according to any one of claims 16 to 22 wherein the vector further includes a second recombination site which is different to the first recombination site.

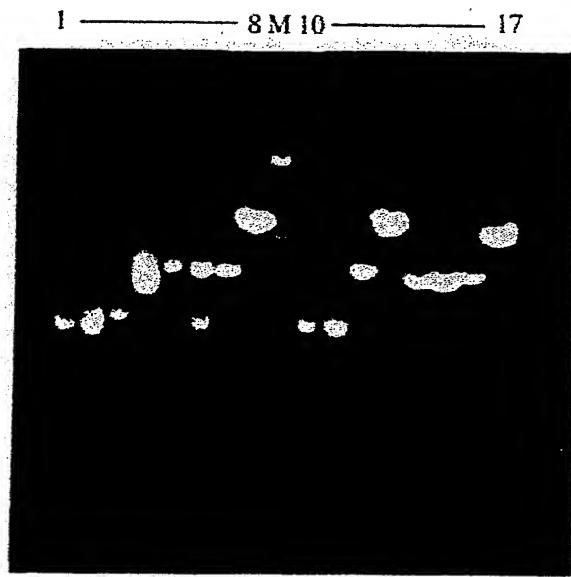
5

24. A phage vector according to claim 23 wherein the second recombination site is a mutant of the *loxP* sequence.

25. A phage vector according to claim 24 wherein the second recombination site is the *loxP511* sequence.

10 26. A phage vector according to any one of claims 16 to 25 wherein the vector further includes

- 15 (a) a sequence encoding a detectable label;  
(b) an inducible stop codon; and  
(c) a sequence encoding an enzymatic cleavage site.



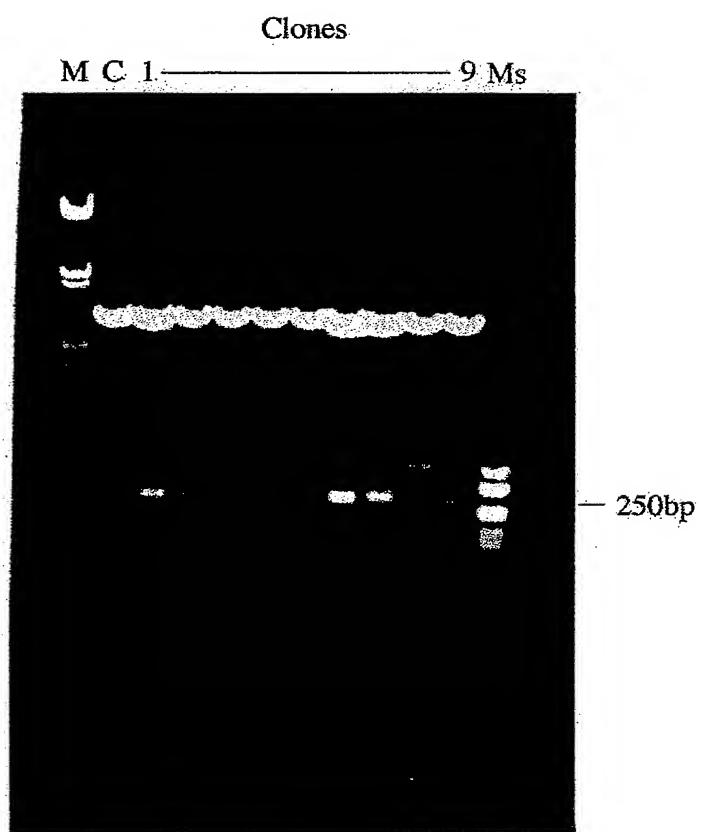
**Figure 1**

2/15



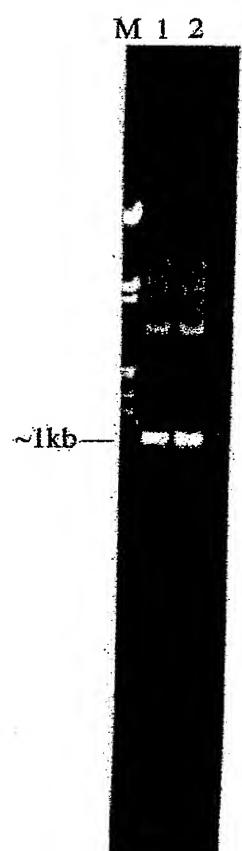
**Figure 2**

3/15



**Figure 3**

4 / 15



**Figure 4**

5/15

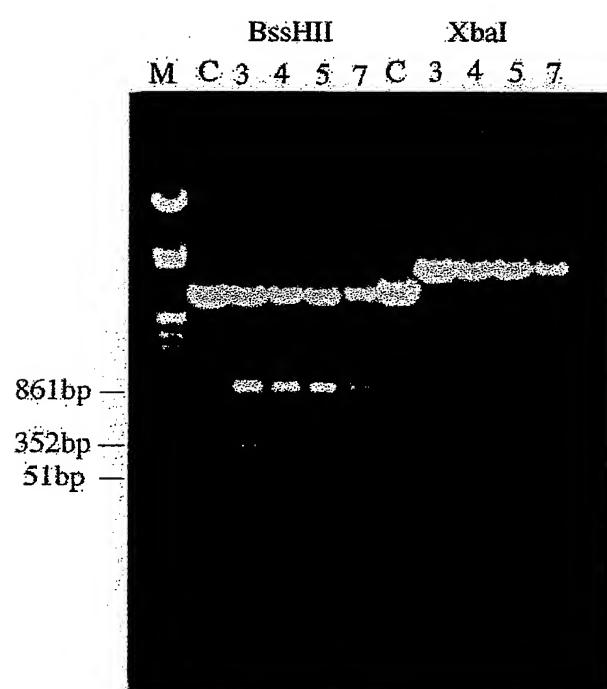


Figure 5

6 / 15

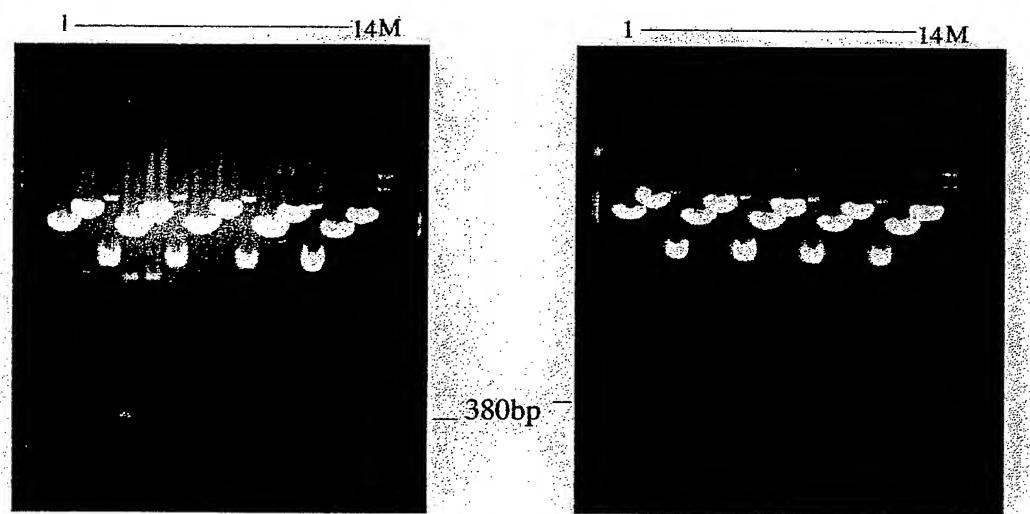


Figure 6

7 / 15

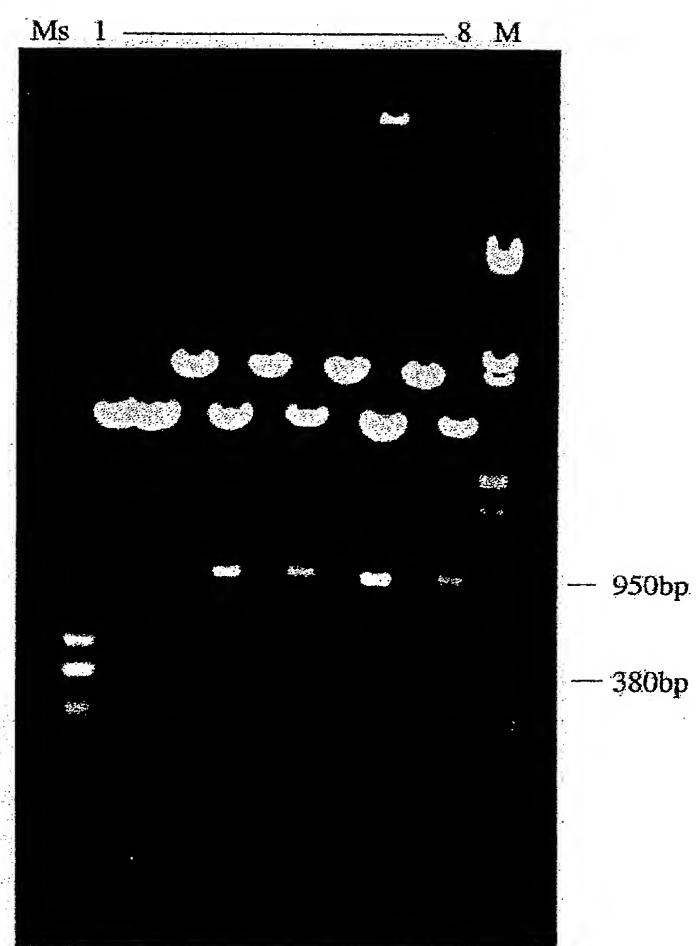


Figure 7

8/15

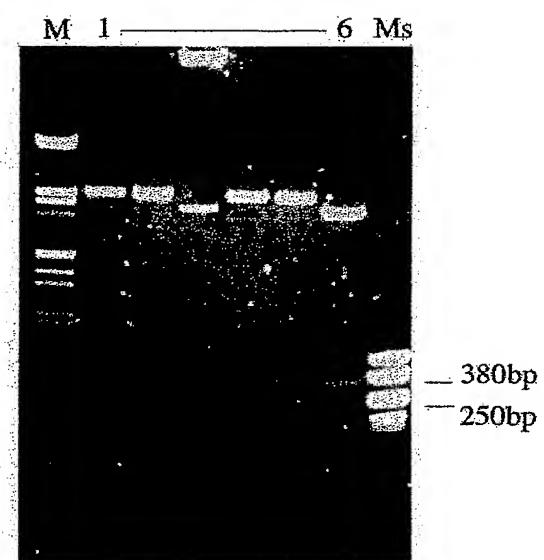


Figure 8

9/15

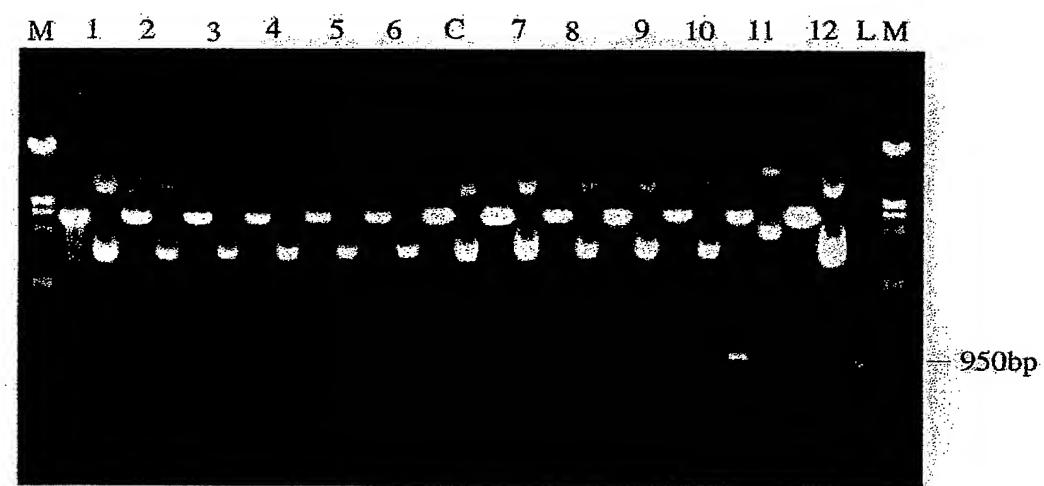


Figure 9

10/15

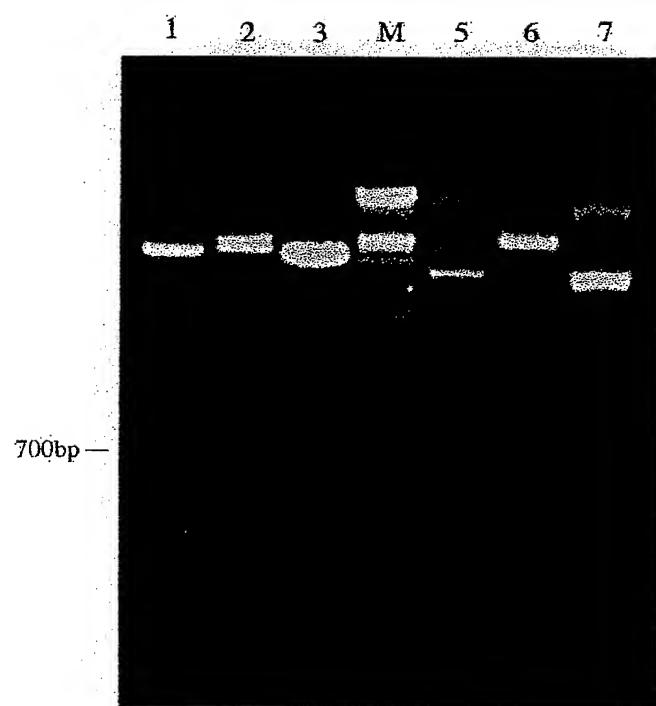


Figure 10

11/15

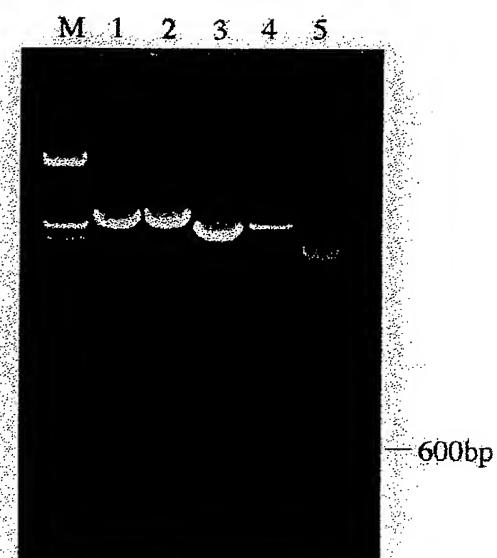


Figure 11

12/15

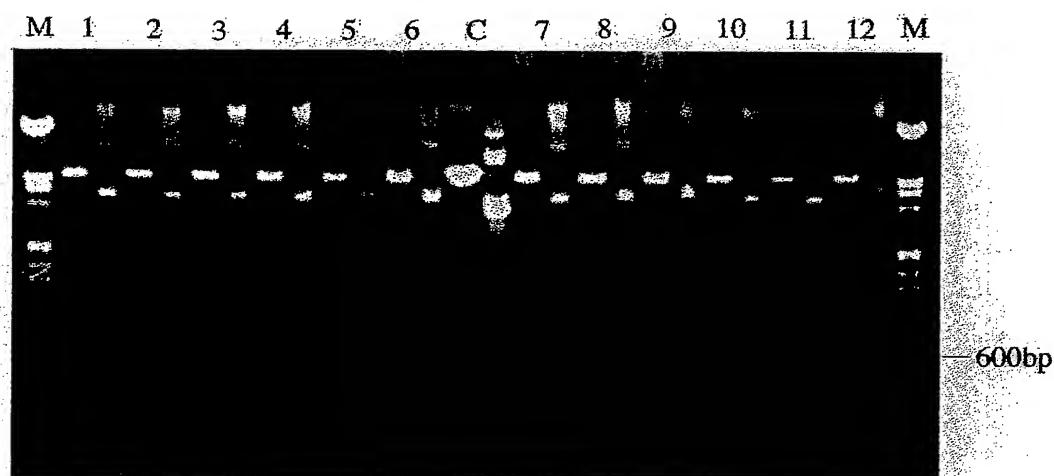
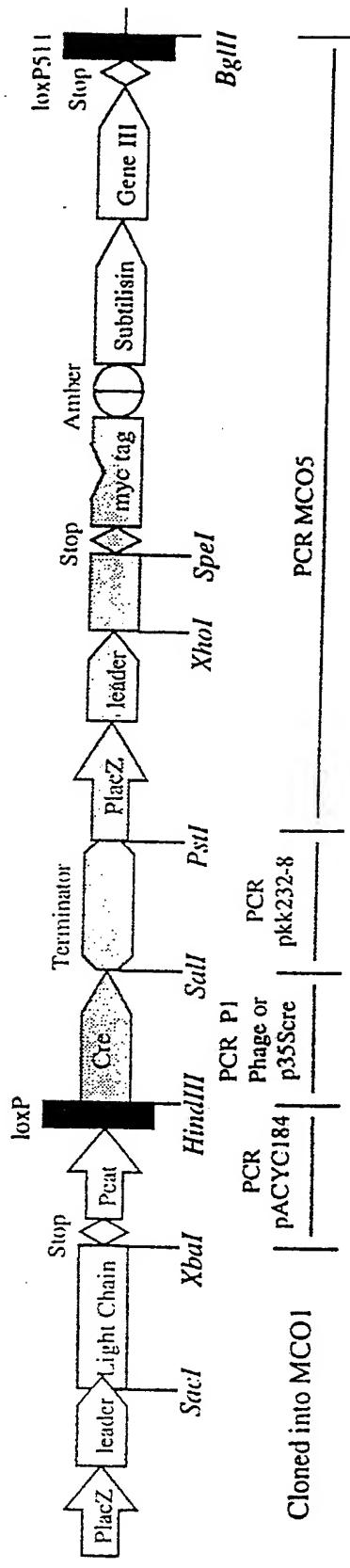


Figure 12

**pMOX****PUX**

13/15

FIGURE 13A

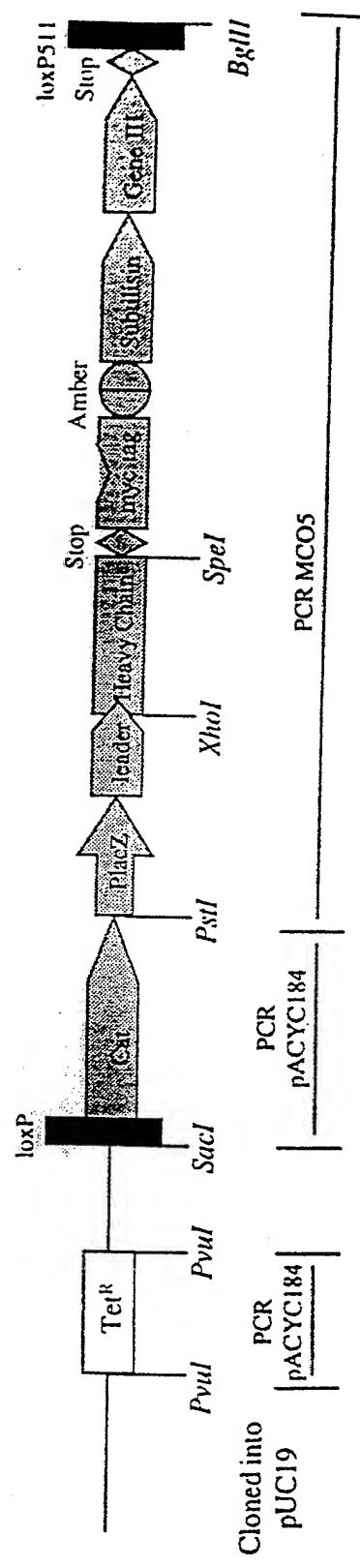


FIGURE 13B

14/15

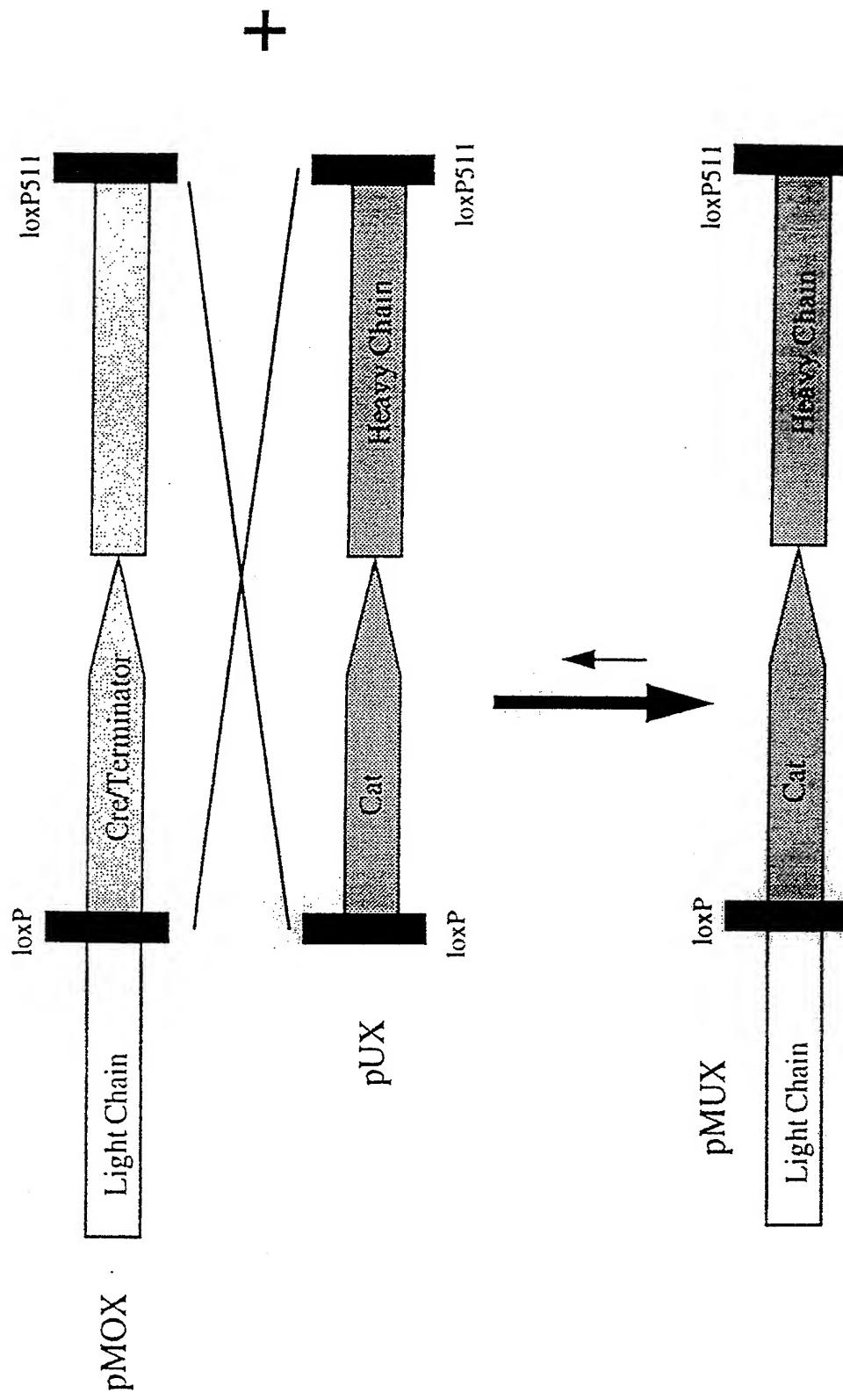


FIGURE 14

15/15

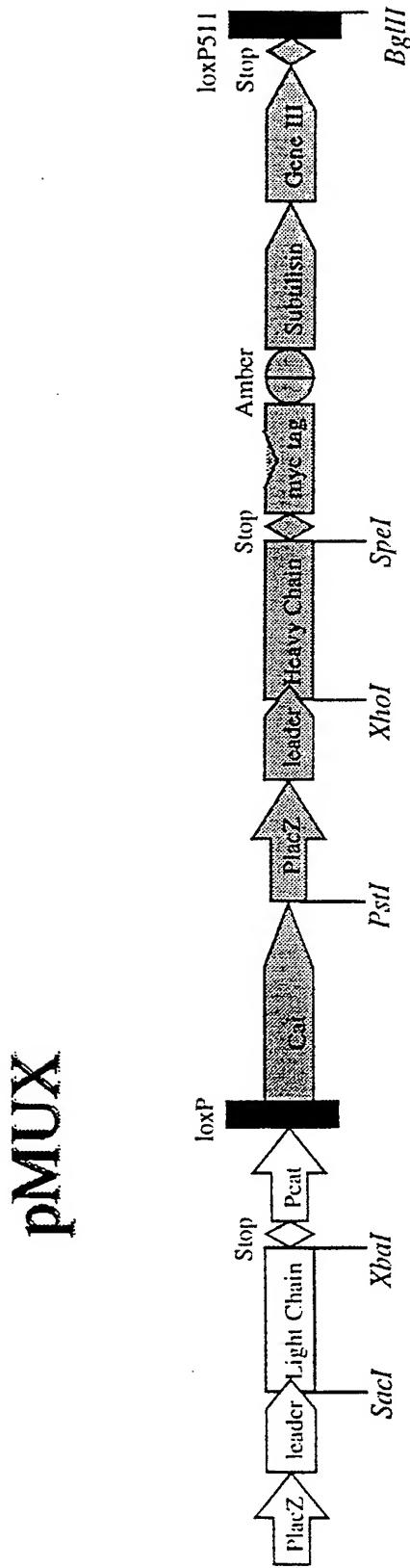


FIGURE 15

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 96/00555

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C12N 15/66, 15/13, 15/70, 15/73		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) IPC: C12N. Chemical Abstracts. Both Through Electronic Databases		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Medline Through Electronic Database		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERWENT Database: WPAT. STN Database: Chemical Abstracts & Medline. Search terms: (cre# or creatin#) (5N) Recombinase#		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X P, Y	TSURUSHITA et al: "Phage display vectors for in vivo recombination of immunoglobulin heavy and light chain genes to make large combinatorial libraries" Gene, 176, pp 59-63, 1996 See entire document	1, 2, 9-12, 14, 16-18 3-8, 13, 19-22
P, X	DAVIES & REICHMANN: "An antibody VH domain with a lox-Cre site integrated into its coding region: bacterial recombination within a single polypeptide chain" FEBS Letters, 377, pp 92-96, 1995 See entire document	1-4, 9, 13, 14, 16-20, 22
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C		<input type="checkbox"/> See patent family annex
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>		
Date of the actual completion of the international search 2 October 1996		Date of mailing of the international search report 29 Oct 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929		Authorized officer  <b>ROBYN PORTER</b> Telephone No.: (06) 283 2318

## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 96/00555

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages <i>(Remove spaces when completed if the page is too long)</i>	Relevant to claim No.
X Y	<p>WATERHOUSE et al: "Combinatorial infection and <i>in vivo</i> recombination: a strategy for making large phage antibody repertoires." Nucleic Acids Research, 21, 9, pp 2265-2266, 1993 See entire article</p>	1-4, 8-14, 16-20, 22-25 5, 7, 15, 21, 26
X	<p>ZOU et al: "Cre-<i>loxP</i>- mediated gene replacement: a mouse strain producing humanized antibodies." Current Biology, 4, 12, pp 1099-1103, 1994 See especially Figure 2</p>	16-20, 22-24